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An investigation of possible allelopathic activity of Artemisia tridentata subspecies vaseyana

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AN INVESTIGATION OF POSSIBLE

ALLELOPATHIC ACTIVITY OF Artemisia

tridentata SUBSPECIES Vaseyana

By

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TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

Allelopathy is chemical inhibition among plants. "Substances which can produce allelopathic effects are...universal in plants and plant communities,...and climax" (1). Tobacco, citrus and walnut trees, sagebrush, and corn are well known examples of plants which produce allelopathic. Also it is known that a variety of plants, e.g., cucumber, barley, peach trees, and grasses respond to allelopathic (2).

Allelopathic compounds belong to several major groups of organic chemicals. These groups include: terpenes, organic acids and aldehydes. Some types of compounds cause indirect allelopathic effects. For instance, indirect effects are shown by antibiotics and enzymes (3). In these cases, microorganisms in the soil are affected by the allelopath and, in turn, the organisms determine the chemical environment of the soil which can be inhibitive (4).

Several methods are used by plants to release allelopathic compounds to the surrounding environment. Rainfall leaches materials from leaves and transfers the compounds to the soil. Because allelopathic are organic compounds, they can be dissolved and concentrated in organic matter, particularly in the liquid phases of soils and sediments in the vicinity (5). Soil adsorption also increases the concentration of allelopathic materials. Ways to introduce possible allelopathic into the environment vary from

Many allelopathic substances are volatile, and are concentrated around the producing plant to form an atmospheric shield, or are washed to the ground by rainfall. Other plants produce inhibitors upon decay: decomposition releases growth-retarding chemicals to the surrounding soil (3,).

Four points should be shown before compounds are established as being allelopathic: 1) the active substance should be isolated and identified, 2) the compound should have allelopathic effects when placed in or in natural crop growth substrates, 3) allelopathic activity should also be tested in field populations, 4) a quantitative relationship between concentration of substance and level of activity should exist (1).

Some contend that the defensive chemicals are root products and only coincidently have inhibitory effects; such chemicals vary too much from plant to plant to be metabolites. Others believe that the compounds are produced explicitly for allelopathic effects. A general overall view suggests that the chemicals were by-products in the plant's evolutionary history but, since they had inhibitory effects on competitors, caused positive selection for the producing plant. Continuation of this process, through time and plant evolution, has resulted in actual defensive chemical production by the plant. Because the by-product protected the plant, it eventually evolved in to a purposeful protectant. Specific glands for production and compartmentalization of protective chemicals in some species are considered evidence that these chemicals are used specifically for defense (1).

Concentration and age contribute to the effectiveness of the allelopathic co[illegible]s. Generally, low concentrations, around 10^{-7} or 10^{-8} molar, are sufficient to produce inhibition. These levels were established [illegible] extract application to [illegible] (7). Soil [illegible] concentrations, [illegible] environmental concentrations, have not been well established. Age of the plant producing the inhibitor and age of the allelopathic extract itself often vary the toxicity. Young plants produce less toxic substances and toxicity increases with age, but extract toxicity decreases with age (3).

While [illegible] these effects are evident in all types of plant communities, the phenomena were first noticed in arid regions. Pioneers [illegible] the inhibitory properties of allelopathy. If [illegible] to germinate, they are in a precarious position to compete for any water. In an arid region seedlings are too inhibited to successfully compete for water and nutrients. In some cases, litter will reduce the germination of allelopathic substances [illegible] and [illegible]. All of these features exhibit in the degree of inhibition of seedling life [illegible].

Allelopathy also [illegible] the availability of nutrients. Soil, a [illegible] root [illegible] plant [illegible] texture, structure, water holding, cation exchange [illegible] nutrient absorption [illegible] are [illegible] affected by [illegible] plants and [illegible] (3,7,10). Allelochemicals could [illegible] specific to particular elements, i.e., acetic, [illegible], etc. Allelopathy may [illegible] not be specific and also self-[illegible] [illegible] on-

stunted. Symptoms of allelopathic interference include chlorosis, early leaf abscission, and darkening and curling of root tips or cotyledons (3).

The germination and successful patterns of plant communities or populations, if not to a degree, by the presence of allelopathic chemicals. Interference effects at the terrestrial or aquatic community. The ecology of land communities is greatly influenced by allelopathic production by a single dominant plant. There exist examples which are limited to the allelopath will become the dominant species (1). Patterns such as farm plots and patches of grasslands are clear evidence of the effects of allelopathic substances.

Allelopathy is known to exist in the aquatic environment. However, if found a viable interest which is not affected by the inhibitory effect, the invader might become resistant.

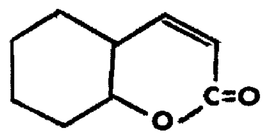
The allelopathic [illegible] of Salvia leucophylla have been studied extensively by [illegible], [illegible], and Muller (1,11,12,13). Based upon the studies of S. leucophylla, they have [illegible] that the [illegible] in the soil, [illegible] the germination of seeds of [illegible], and [illegible] other, with Salvia [illegible]. Muller et al., determined that allelopathic [illegible] both the allelopathic mechanism [illegible] to determine if there could be certain [illegible] for the allelopathy

in the field. [illegible] transporting [illegible] volatile [illegible] experiments were carried out to see if the soil would concentrate volatile [illegible].

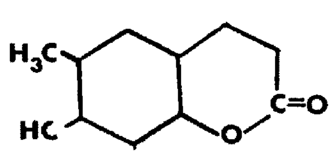
[illegible] that [illegible] of P. [illegible] could be [illegible] in the soil, but [illegible] definitely determined [illegible] played a major role in [illegible] of the [illegible] from the [illegible] in the soil (1 ,11,1 ,1).

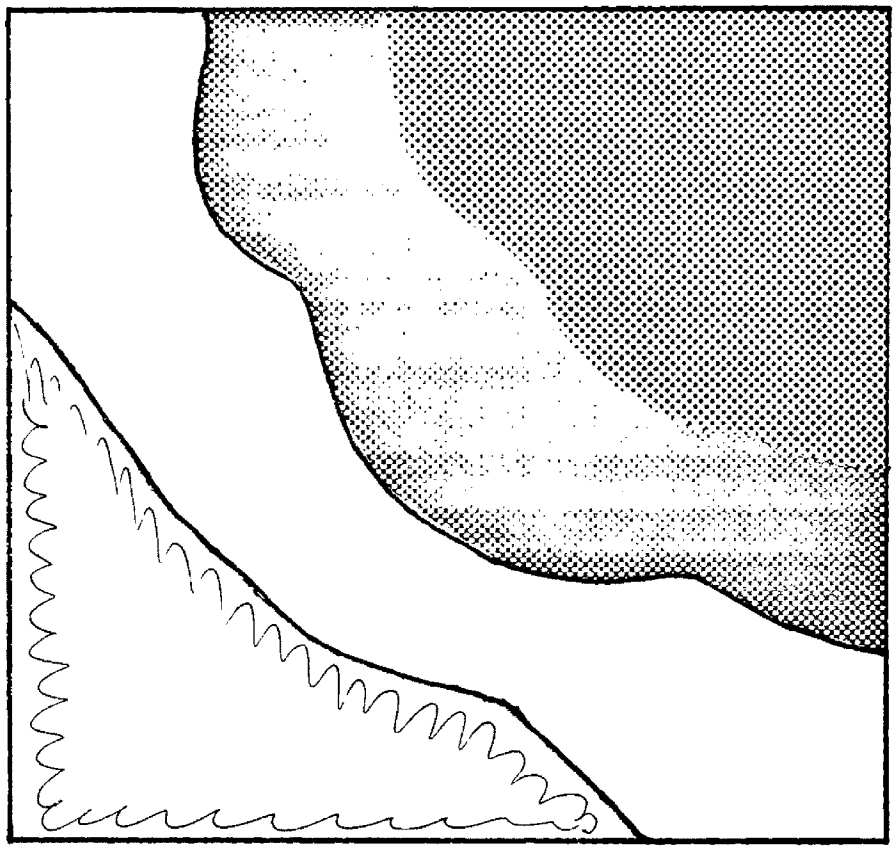
[illegible] altitude region [illegible] exhibit the same general pattern [illegible], Fig. 1 ( ). A [illegible] center outward. [illegible] the [illegible]. [illegible] the center, [illegible].

[illegible] nucleus:



Scopoletin [illegible]





Deteriorating Shrub Stand

Vigorous Growth

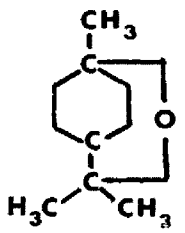
Bare Area

Native Grassland Community

Figure 1. Growth pattern exhibited by some aromatic shrubs including chapparal and sagebrush.

another coumarin found in *Artemisia*, scoparia, has been shown to inhibit chromosome condensation in onion (1). [illegible] fluoresce in ultra violet light and can be extracted by steam distillation. Purification and identification can be accomplished by several chromatographic methods (15).

[illegible] oil of *Artemisia* [illegible] the [illegible] of the [illegible] odors, oils from *Artemisia* have been used in paints, foods, flavorings, [illegible] and medicines. [illegible] or other studies (16). Some therapeutic uses have been shown to be due to terpenoid components. A monocyclic monoterpene is shown in:



Essential oils usually contain one or two predominant terpenoids. However, oils are complex mixtures; therefore, separation of individual components is difficult. Extraction involves steam distillation, solvent extraction by saponification with alkali, and aeration (15). Separation methods include: chromatography, i.e., gas, reverse phase, thin layer, column; and derivative formation (15).

Since *Artemisia* exhibits some resistance to the use of *S. leucoblia*, the author studied to extract allelochemic compounds which might be present in the plant using known bioassay techniques.

The initial objective of this study was to investigate the toxic effect of *Artemisia tridentata* var. *vaseyana* on growth of

Cucumis sativus, Avena and three native herbs. One native herb was collected from within the A. tridentata community, a second was taken from the ecotone of an A. tridentata community, and a third was collected from an area in which A. tridentata growth was absent. Toxic effects of macerated leaf material, leaf extract, and purified growth inhibitors were quantified in seed growth chambers. The toxic or stimulatory effects on germination, radicle and hypocotyl growth, respiration and photosynthesis were determined.

However, neither Avena nor the native herb seeds could be successfully germinated; therefore, the section of the proposal concerning these species could not be developed. A study of autotoxic effects on viable A. tridentata ssp. wyomingensis seeds replaced this section. Photosynthesis studies were not carried out since seeds that were germinated and grown in dark chambers were chlorotic.

It was hoped that these tests would reveal the presence or absence of allelopathic compounds in A. tridentata ssp. vaseyana.

CHAPTER II

MATERIALS AND METHODS

Experimental Plants

Cucumis sativus, Boston pickling cucumber from the Chas E. Lilly company, was used because of its rapid and uniform germination. After soaking in aerated, distilled water for one and one-half to two and one-half hours, seeds were placed in seed chambers. These chambers were 300 ml Erlenmeyer flasks in which filter paper was placed over a layer of glass wool. Seeds were placed on the filter paper and 20 ml distilled water were added to the flasks. Flasks were sealed with parafilm and placed in the dark at approximately 26°C for four to six days. Growth inhibition was obtained by placing various quantities of macerated sagebrush in vials in the flasks or by placing various quantities of the different extracts or purified compounds directly on the filter paper in the flasks.

Artemisia tridentata ssp. vaseyana was gathered six miles west of Missoula, Montana, between May and September of 1971. Leaves and a few buds were picked from the gathered sprigs and then macerated. Those that were picked and macerated immediately after gathering were labeled fresh and those that were first dried for several weeks after gathering, and then picked and macerated were labeled dry.

Fractionation and Purification Procedures

Extraction

The extraction procedure was as follows. First, 0.5[illegible] kg of macerated, dried s[illegible]gebruch was extracted with 2.[illegible] l of 95[illegible] ethanol. The material was soaked in the ethanol for one day and then filtered. A second portion of 1.8 l of [illegible]% ethanol was added to the once extracted mash. The two extract filtrates were evaporated to dryness to yield 114 g of dark green, syrupy residue. Then, 100 g of this residue was partitioned between 200 ml distilled water and 750 ml chloroform. The chloroform portion was washed with 200 ml water. The water portion and wash were combined and evaporated to dryness giving approximately 30 g gold-brown syrup. Upon evaporation, the chloroform portion yielded 50 g very dark green, oily residue which was partitioned between 500 ml aqueous methanol (1 water: 9 methanol), and five 200 ml portions of petroleum ether, 30 - 110°C fraction. Each of these was evaporated; the aq. MeOH portion yielded 30 g dark green syrup and the petroleum ether portion yielded 22 g of a green, slightly viscous solution. Part of the aq. MeOH portion was used for chromatography.

Column Chromatography

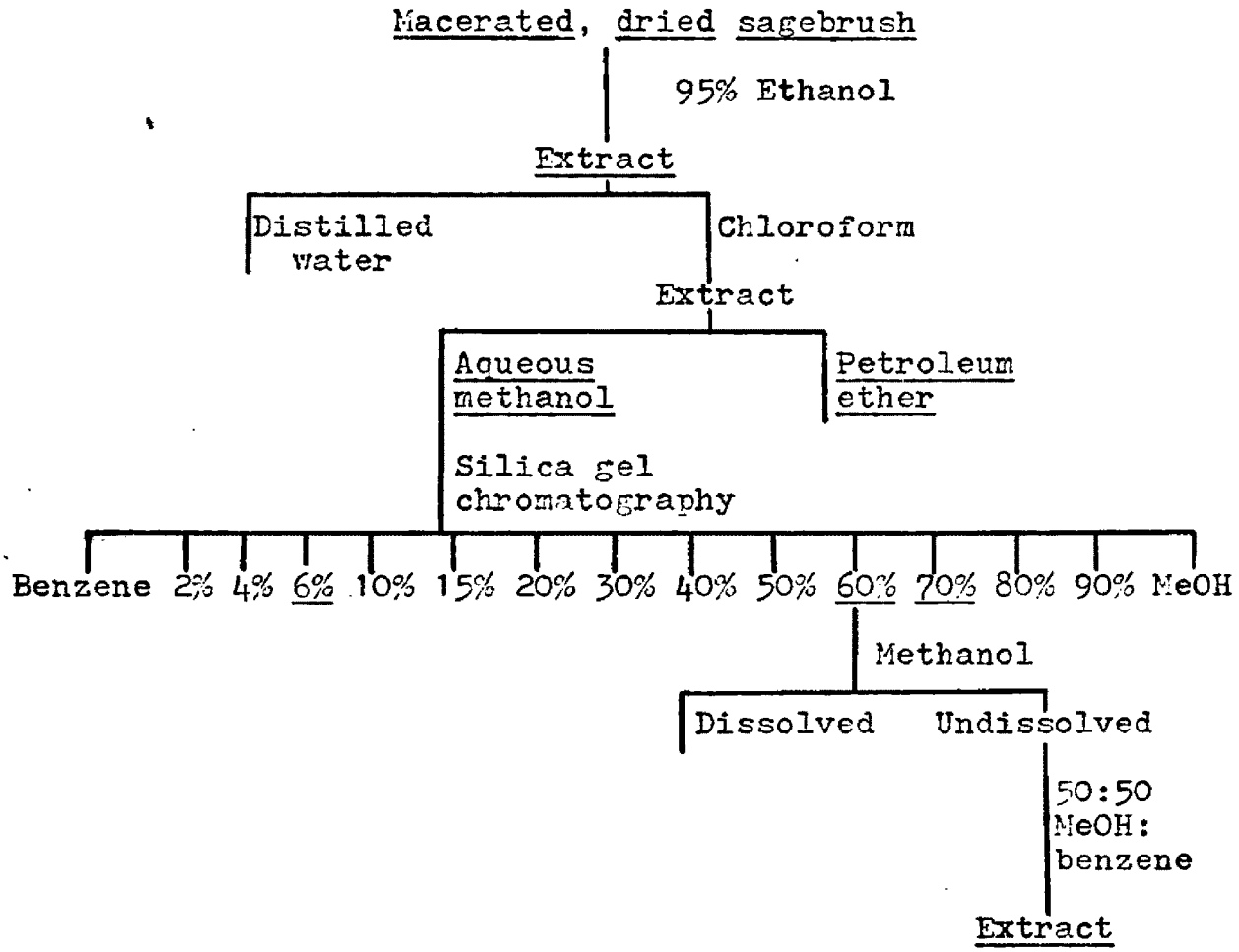
A chromatography column was prepared using 200 g powdered silica gel slurried in benzene. To this column was added 25 g of the aq. MeOH extract dissolved in 10 ml benzene. Solvents for elution were mixtures of methanol and benzene which varied in percentage by volume composition. Each solvent was passed through the column until evaporation of the eluant gave no product.

Successive elutions and evaporation of the fractions gave the following products: 17.1 l 10% benzene yielded 7.0 g dark green residue; 4.3 l 2% MeOH in benzene yielded 2.[illegible] g dark green residue; 6.2 l 4% MeOH-benzene yielded 1.5 g dark green, very viscous, oily residue; 5.3 l 6% MeOH-benzene yielded 1.[illegible] g dark green, viscous residue; 7.4 l 10% MeOH-benzene yielded 1.5 g dark green, crystalline and oily residue; 7.6 l 15% MeOH-benzene yielded 1.5 g green-brown, crystalline and syrupy residue; 7.1 l 20% MeOH-benzene yielded 1.[illegible] g brown crystalline residue; 11.1 l 30% MeOH-benzene yielded [illegible] g brown, crystalline residue and yellow-gold syrup; 6.[illegible] l 40% MeOH-benzene yielded 0.6 g dark brown, oily, crystalline material; 4.1 l 50% MeOH-benzene yielded 0.[illegible] g dark yellow, syrupy, crystalline residue; [illegible] l 60% MeOH-benzene yielded 0.[illegible] g brown crystals; 10.5 [illegible] 70% MeOH-benzene yielded 0.2 g dark yellow powder; 7.3 l 80% MeOH-benzene yielded 0.1 g light brown crystals; 4.2 l [illegible]% MeOH-benzene yielded 0.05 g dark yellow powder; finally, 4.4 l 100% MeOH yielded 0.05 g brown powder. After activity tests, the 60% MeOH-benzene fraction from this column was selected for further purification.

The 60% MeOH-benzene fraction was partially dissolved in 100% MeOH. The dissolved portion was chromatographed on a [illegible] g silica gel chromatography column and separated into a diffuse band. Thin layer chromatography showed that different tubes of the band contained one and the same compound. The undissolved portion was dissolved in 50:50 MeOH:benzene, filtered and chromatographed as before. A flow chart covering extraction, column fractionation and purification is presented in Table 1.

Table 1. Flow chart following the extraction of active inhibitors present in Artemisia tridentata ssp. vaseyana. Those fractions underlined were found to be biologically active as inhibitors. X% expresses percentage of methanol in benzene

EXTRACTION METHOD



Thin Layer Chromatography

The 70% MeOH-benzene fraction and both the dissolved and undissolved portions of the 60% MeOH-benzene fraction were compared with pure esculin using thin layer chromatography. Thin layer chromatography chambers contained 70:30 MeOH:benzene, 60:40 MeOH:benzene, and 50:50 MeOH:benzene as solvent systems. Sheets of Silica Gel II-F from Baker-flex™ were used as the chromatography matrix.

Melting point determinations were attempted using a Fisher-Johns M. P. apparatus. An attempt to obtain a nuclear magnetic resonance spectrum for the undissolved 60% MeOH-benzene fraction was made using the Varian model A-60 II, d-chloroform solvent and TMS lock.

Chromatography indicated that the undissolved portion of the 60% MeOH-benzene fraction was not esculin. The material fluoresced under ultraviolet light and had R_f values: 0.60 in 60:40 MeOH:benzene and 0.33 in 70:30 MeOH:benzene. NMR work showed an inconclusive broad band at 2.7 - 3.5 τ; M. P. determinations gave broad ranges. Amount of compound limited analytical measures. The 70% MeOH-benzene fraction did not fluoresce and its thin layer chromatography and NMR differed from that of the 60% MeOH-benzene fraction.

Germination and Growth Measurements

Germination percentage was figured on the assumption that any plant with its radicle extended from the seed pod was a germinated plant. Such plants were counted and compared to the number of-

minated in a control chamber.

Growth measurements included length of radicle and hypocotyl, and number of lateral roots. Radicles were measured from "peg" to tip; hypocotyls were measured from "peg" to the point of attachment of the cotyledons.

Measurements of germination, growth and respiration recorded those effects produced by: leaves, crude extracts, the column fractions separated by chromatography and pure coumarin. Macerated sagebrush leaves (fresh and dried), the aqueous methanol extract and pure coumarin were used in varying amounts in seed chambers. One specific test measured the effects of sagebrush on seedlings of different ages. Germination and growth measurements were also carried out on the dissolved and undissolved portions of the 6" filter-bench extraction. For all of the previous trials A. sativa was the assay species. A. tridentata and Lycopersicon seeds were used to test antitoxic activity of the aq. MeOH fraction.

Manometric Techniques

Measurement of respiration employed a Gilson respirometer. Respirometer flasks contained 0.2 g fresh weight of seedlings, 1 ml diethanolamine buffer solution, 0.4 ml in the center well and 0.3 ml in the side arm (17), which maintained a constant CO_2 level, and in some cases various amounts of fresh sagebrush. Primarily, the respiration of seedlings was measured without sagebrush present in the respirometer flasks. Gas exchange was measured in microliters at five or ten minute intervals. The bath temperature was kept at 25°C in all experiments reported.

CHAPTER III

BUSH TEA

Macerated *A. tridentata* leaves were tested in order to establish whether sagebrush produces biologically active toxins. Measurements in the following tables, 2 through 10, used corrected means for average lengths. Range indicates the spread of formed clusters rather than the spread of all germinated seedlings; ungerminated seeds were eliminated from calculations. "Number of sample/ number planted" recorded the number of samples which formed the cluster compared with the number of available values (number planted). Activity is based on the percentage of growth a group of plants has when compared to a control group.

Leaves were macerated in order to release volatile compounds more readily and for convenience in handling. Generally, fresh material showed inhibitory activity on growth, Table 2. Germination was not significantly affected by 0.1 g to 5.0 g of leaf material. Stimulation of growth resulted from 0.1 g material with hypocotyl growth showing the greatest increase and radicle growth showing the least. Severe inhibition occurred when 0.5 g to 5.0 g of sagebrush was present in growth chambers. Growth decreased with increasing amounts of leaf material. Hypocotyls showed most resistance to inhibition, but even in the case of hypocotyls, when 0.5 g of sagebrush was present, growth was reduced to 23% of the control. Radicle growth was cut to 3.5% of the control with 0.5 g

sagebrush. However, 1.[illegible] reduced growth to 1.6% of the control, while 5.0 g sagebrush reduced growth to only 5.[illegible] of the control. This is the only deviation from a strictly proportional relationship between reduced growth and increased amounts of leaf material. No lateral roots were developed on the main root when 0.5 g to 5.0 g of sagebrush was present.

Table 2. Growth of G. aptivus seedlings exposed to fresh macerated A. tridentata leaves. Total germination time was 6 days.

Fresh macerated leaf material, g	0	0.1	0.5	1.[illegible]	5.0
Number of seeds germinated	2[illegible]	1[illegible]	[illegible]	2[illegible]	1[illegible]
% of Control	--	95.[illegible]	100	1[illegible]	95.0
Radicle length, mm					
Average	[illegible]7	67	[illegible].[illegible]	1.[illegible]	1.0
Range	38-84	53-75	1-[illegible]	1	1-[illegible]
Number of seedlings/ number planted	15/20	12/19	13/2[illegible]	1[illegible]/2[illegible]	20/20
% of Control	--	1[illegible]6	5.5	1.6	3.0
Hypocotyl length, mm					
Average	[illegible]0	32	5.[illegible]	4.[illegible]	0.0
Range	1[illegible]-[illegible]0	[illegible]5-3[illegible]	[illegible]-1[illegible]	3-[illegible]	0
Number of seedlings/ number planted	17/[illegible]	12/1[illegible]	20/20	15/2[illegible]	20/20
% of Control	--	1[illegible]5	2[illegible]	1[illegible]	[illegible].0
Number of lateral roots					
Average	15	1[illegible]	[illegible].[illegible]	[illegible].[illegible]	[illegible].[illegible]
Range	[illegible]-2[illegible]	1[illegible]-2[illegible]	[illegible]	[illegible]	[illegible]
Number of seedlings/ number planted	1[illegible]/[illegible]0	1[illegible]/1[illegible]	17/[illegible]0	[illegible]0/2[illegible]	20/2[illegible]
% of Control	--	1[illegible]	[illegible].	[illegible].[illegible]	0.0

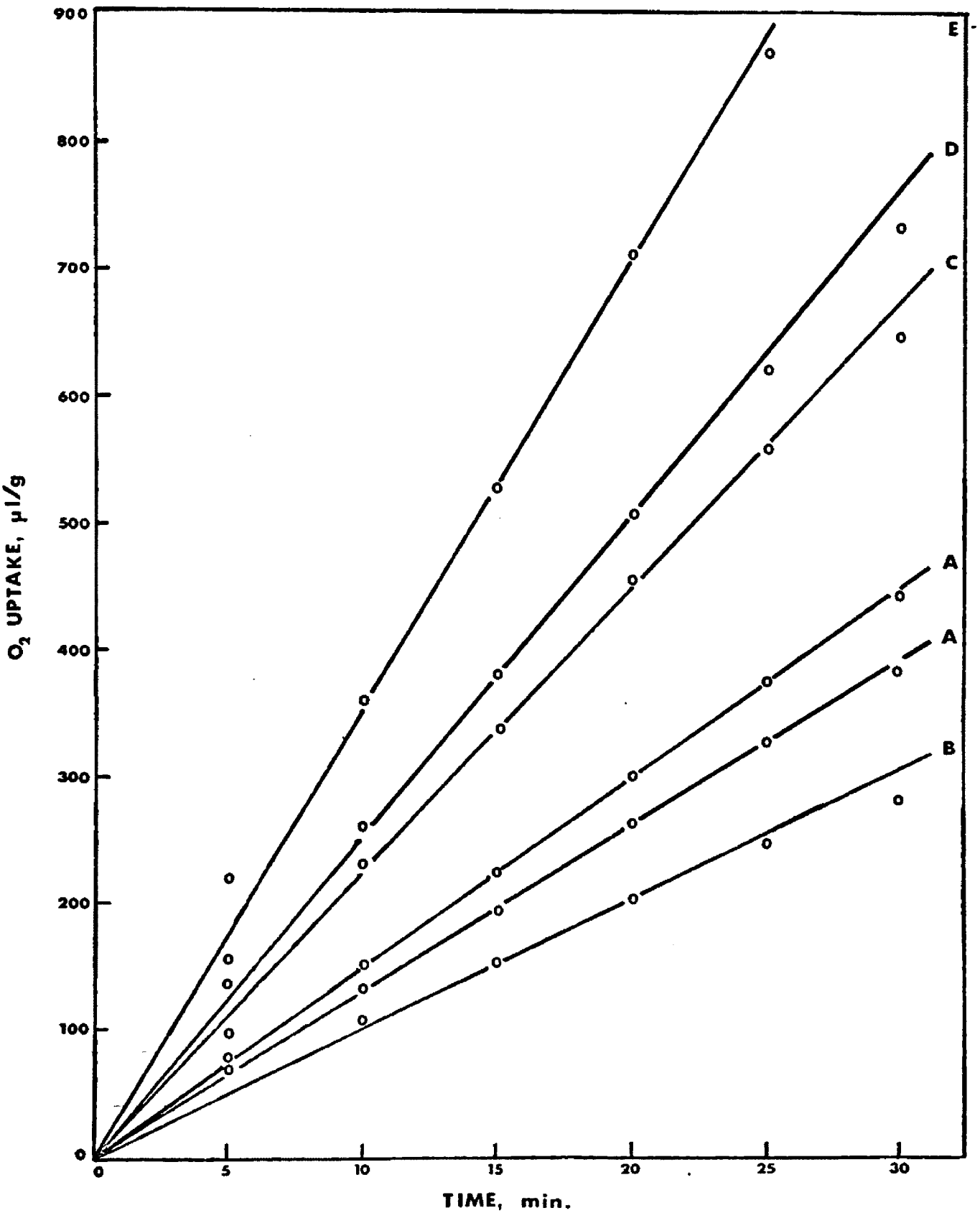
Since cineole and camphor are known inhibitors (10), and it is known that they are present in A. tridentata, dry sagebrush was tested to see if the maceration was still biologically active after these allelopaths had volatilized. Dry material was also [illegible] easily obtained

Dry leaf macerate proved inhibitory, however, the degree of inhibition was less than that of fresh material, Table 7. Germination was not affected. A very slight effect on growth was caused by 0.1 g dry material. Inhibition of growth increased as the amount of inhibitory material present increased from 0.5 g to 5.0 g. Hypocotyl growth was reduced slightly, radicles were more severely affected and lateral roots were inhibited the most with no root extension when 1.0 g and 5.0 g macerate were present. Seedlings grown in the presence of 0.5 g to 5.0 g of sagebrush exhibited stimulation of geotropism, Fig. 2. Germination was inhibited when seedlings were grown with 0.1 g of sagebrush.

Table 7. Growth of C. sativus seedlings grown in dry macerated A. tridentata leaves. Total germination time was 6 days.

Dry macerated leaf material, g	0	0.1	0.5	1.0	5.0
Number of seeds germinated	20	20	20	20	20
% of Control	--	100	100	100	100
Radicle length, mm					
Average	63	66	38	4.0	1.6
Range	53-84	54-74	13-25	2-10	1-3
Number of samples/ number planted	19/20	13/20	11/20	10/20	17/20
% of Control	--	105	31	6	2.5
Hypocotyl length,					
Average	22	21	9.2	7.2	3.4
Range	15-30	6-33	5-15	4-16	2-5
Number of samples/ number planted	17/20	20/20	14/20	15/20	13/20
% of Control	--	95	41	32	16
Number of lateral roots					
Average	15	16	4.1	1.1	0.0
Range	9-25	13-19	0-11	0-3	0
Number of samples/ number planted	16/20	15/20	15/20	11/20	20/20
% of Control	--	107	27	7.3	0.0

Figure 2. Rate of oxygen uptake by excised root-tips of Pseudotsuga menziesii as affected by various amounts of dried, ground Artemisia tridentata. Tips were excised from 6-day old seedlings. A - control; B - 0.1 g; C - 0.5 g; D - 1.0 g; E - 5.0 g sagebrush.



Since dry leaf material was toxic, extracts from it were prepared. The 95% ethanol extract and subsequent water, petroleum ether and aqueous methanol extracts were tested for allelopathic activity. In Table 4, the ethanol, pet ether and aq. MeOH extracts showed inhibitive biological activity on radicle growth. The water extract was extremely stimulatory, causing radicle growth to be 137% of the control. Hypocotyl and lateral root growth were completely inhibited by all extracts. The water extract and the aqueous methanol extract affected germination, the aq. MeOH being the most inhibitory.

Table 4. Growth of *L. sativus* seedlings exposed to various extracts, solvents evaporated. Total germination time was 5 days.

Types of solvents (0.1 g extract)	Control	EtOH	H_2O	pet ether	aq. MeOH
Number of seeds germinated	19	19	17	20	15
% of Control	--	100	89.5	105	78.9
Radicle length, mm					
Average	6.4	1.0	12	1.0	1.0
Range	1-12	1	1-24	1	1
Number of sample/ number planted	17/20	19/20	17/17	14/20	14/15
% of Control	--	16	187	16	16
Hypocotyl length, mm					
Average	2.0	0.0	0.0	0.0	0.0
Range	0	0	0	0	0
Number of sample/ number planted	16/20	20/20	12/17	20/20	15/15
% of Control	--	--	--	--	--
Number of lateral roots					
Average	0.0	0.0	0.0	0.0	0.0
Range	0	0	0	0	0
Number of sample/ number planted	11/17	2/20	12/17	2/20	15/15
% of Control	--	--	--	--	--

Because it was the most effective extract, the aq. MeOH fraction was subjected to tests to relate amount of extract present to amount of inhibition produced. In all cases from 0.01 g to 0.1 g, except that of 0.07 g, inhibition increased as the amount of extract increased. Inhibition occurred in all cases, and reached maximum inhibition in the presence of 0.1 g aq. MeOH extract. In general, the growth of radicles was inhibited least. However, considering the case of 0.07 g material, results differed from the general pattern. This exception was regarded as experimental error rather than a deviation of significance. Germination was not significantly affected by the presence of these amounts of aqueous methanol extract, Table 5. In general, respiration was stimulated by the aq.

Table 5. Growth of *G. sativus* seedlings exposed to various amounts of aqueous methanol extract. Total germination time was 5 days.

Aq. MeOH extract, g	0	0.01	0.03	0.05	0.07	0.10
Number of seeds germinated	10	10	10	9	9	10
% of Control	--	100	100	90.0	90.0	100
Radicle length, cm						
Average	21	20	2.0	1.4	2.3	1.0
Range	42-116	15-30	2-4	1-2	1-4	1
Number of sample/ number planted	7/1	7/10	6/10	7/10	7/10	3/10
% of Control	--	95	5.0	2.0	5.0	1.4
Hypocotyl length, cm						
Average	55	30	3.1	0.0	4.8	0.0
Range	30-67	11-47	0-3	0	0-14	0
Number of sample/ number planted	6/10	10/10	7/10	6/10	10/10	3/10
% of Control	--	55	6.0	0.0	1.5	2.0
Number of lateral roots						
Average	26	13	0.0	0.0	1.0	0.0
Range	02-30	11-14	0	0	0-11	0
Number of sample/ number planted	5/10	6/10	5/10	6/10	1 /10	10/10
% of Control	--	50	0.0	0.0	15	0.0

MeOH extract, Fig. 3. In all cases where growth was inhibited respiration was stimulated. However, amount of growth inhibition was not proportional to the amount of increase in the rate of respiration.

Tables 6 and 7 present data concerning biological activity of the chromatographic column fractions of the crude methanol extract. This extract was chosen for chromatography because of its high biological activity. Column fractions were tested to determine which showed inhibitive properties. The 1% and 10% MeOH-benzene fractions were more active of those presented in Table 6. Inhibitory activity [illegible] from the 2% through to the

Table 7. Growth of *L. sativus* seedlings treated to various chromatography column fractions, 5 mg. Total germination time was 6 days.

Column fraction (% MeOH-benzene)	Control	2%	4%	6%	10%	15%	25%
Number of seeds germinated	10	10	[illegible]	10	10	10	[illegible]
% of Control	--	100	[illegible]	100	100	100	[illegible]
Radicle length, mm							
Average	100	101	97	68	33	42	117
Range	91-110	91-114	[illegible]-111	[illegible]	[illegible]	21-52	[illegible]
Number of seeds/ number planted	10/10	10/10	9/10	10/10	9/10	6/10	9/10
% of Control	--	97	93	57	78	61	107
Hypocotyl length, mm							
Average	[illegible]	77	[illegible]	62	[illegible]	[illegible]	87
Range	69-89	64-94	51-[illegible]	49-71	55-61	61-111	71-[illegible]
Number of seeds/ number planted	[illegible]/10	10/10	9/10	9/10	6/10	9/10	9/10
% of Control	--	110	[illegible]	[illegible]	75	117	107
Number of lateral roots							
Average	[illegible]	22	17	11	14	12	19
Range	18-[illegible]	[illegible]-28	[illegible]-[illegible]	10-[illegible]	[illegible]-17	[illegible]-19	17-[illegible]
Number of seeds/ number planted	9/10	10/10	9/10	6/10	10/10	9/10	9/10
% of Control	--	110	[illegible]	73	64	57	92

Figure 3. Rate of oxygen uptake by excised radicles of Fucus spiralis as affected by various amounts of aqueous methanol extract residue in solution. Radicles were excised from 5-day old seedlings. A - control; B - 0.01 g; C - 0.03 g; D - 0.05 g; E - 0.07 g; F - 0.10 g residue.

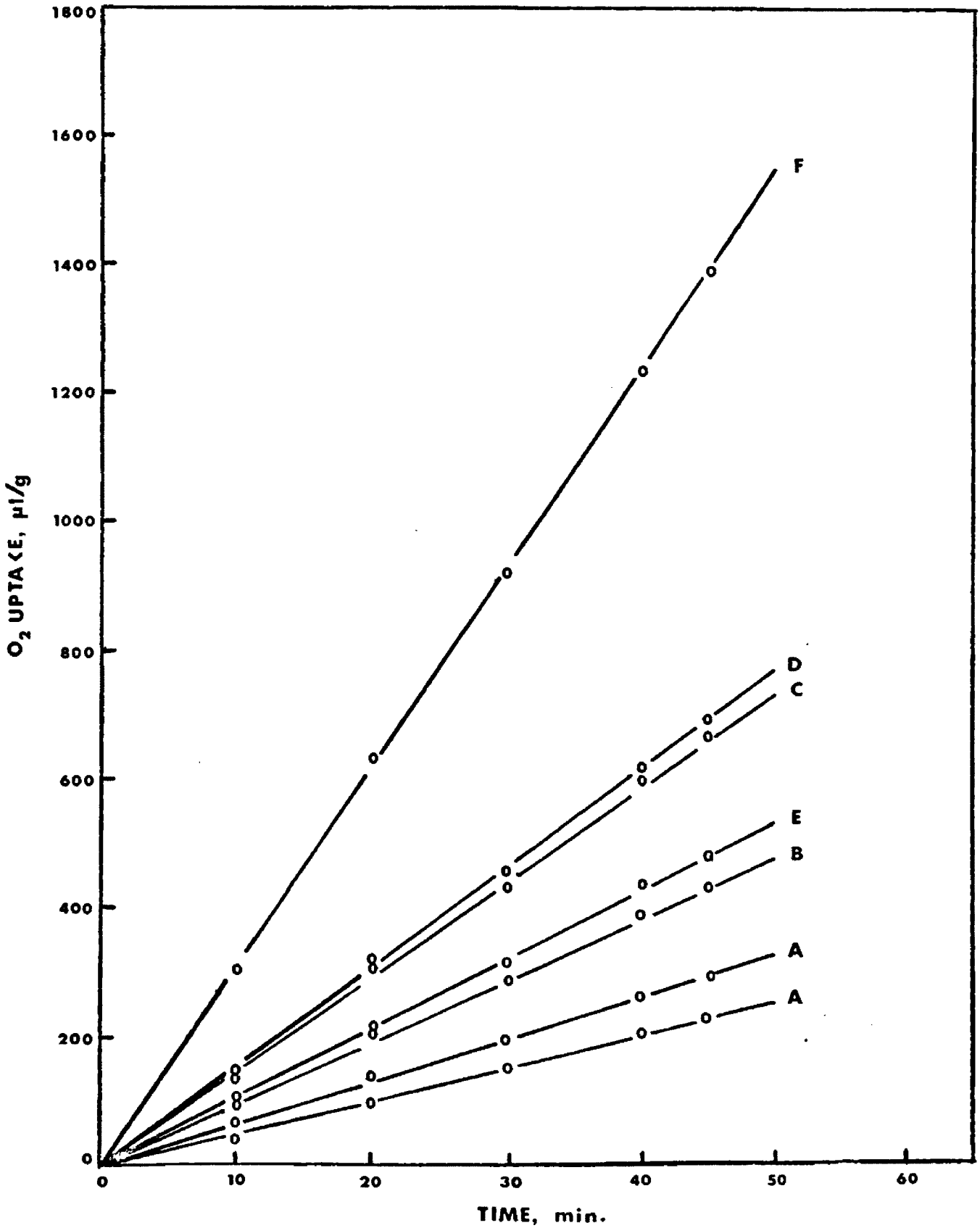


Table 7. Growth of *C. sativus* seedlings exposed to various column fractions, 10 mg. Total germination time was 4 days.

Column fractions (% MeOH-benzene)	Control	10%	15%	20%	30%	40%	50%	60%	70%	80%	90%
Number of seeds germinated	9	10	10	10	10	10	10	10	10	10	10
% of Control	--	110	110	110	110	110	110	110	110	110	110
Radicle length, mm											
Average	69	80	81	95	79	71	68	71	50	89	79
Range	51-85	73-89	75-85	85-104	62-103	57-85	52-82	61-84	37-61	83-92	71-91
Number of sample/ number planted	8/9	8/10	7/10	8/11	10/10	10/10	10/10	9/10	10/10	5/10	8/10
% of Control	--	116	117	138	114	103	98	103	72	129	114
Hypocotyl length, mm											
Average	30	42	30	39	21	39	30	19	17	33	38
Range	25-42	35-49	27-34	17-50	17-26	35-44	27-31	13-25	8-29	24-50	36-40
Number of sample/ number planted	9/9	7/10	7/10	11/11	5/10	7/10	8/10	8/10	10/10	10/10	5/10
% of Control	--	140	100	130	70	130	100	63	57	110	127
Number of lateral roots											
Average	15	18	13	15	16	17	13	15	13	17	14
Range	12-19	12-22	10-17	7-22	11-17	15-19	11-15	12-16	9-17	12-21	11-16
Number of sample/ number planted	8/9	9/10	8/10	11/11	9/10	8/10	10/10	6/10	10/10	10/10	8/10
% of Control	--	120	87	100	107	113	87	100	87	113	93

60' and 70' fractions and then decreased to the 80' fraction. There was no net math on concentrating effect on specific dry sugars. In Table 7 it is shown that the 60' and 70' [illegible] fractions exhibited the greatest inhibitory activity, while the 50' and 80' fractions exhibited stimulatory activity. Hydrolysis influence patterns exhibited generalized increases in plant organ selectivity. Epicotyl growth was not inhibited.

As a result of the tests on column fractions, the 60', 70' and 75' Bio-Gel-because fractions were carried to exhibit active, inhibitory components. It was also assumed that the 60' and 70' fractions might contain the auxin activity. Therefore, these two fractions were subjected to further analysis including refinement of the 60' fraction (not rigorously obtained). As shown in Tables 8 and 9, the undissolved portion of the 60' fraction had inhibitory properties, while the dissolved portion showed no kind of activity. The 70' fraction exhibited relative inhibitory activity. The undissolved portion of the 60' Bio-Gel-because fraction, lateral root growth was most resistant to activity, and exhibition stimulation. Hypocotyls were not inhibited. Root lateral growth was not affected by the 70' Bio-Gel-because fraction, however, radicle growth was reduced to 60 - 75% of the control and hypocotyl growth was reduced to around 80 - 85% of the control. These results, plus the analytical procedure as described for the next right-hand section, suggested that the 60' and 70' fractions do not contain the auxin compound.

Isolation and analytical procedures indicated that the active

Table 3. Growth of C. sativus seedlings exposed to refined 50% MeOH-benzene fraction and 70% MeOH-benzene fraction. Total germination time was 4 days.

Treatment	Control	Undissolved portion (50% fraction, 1 mg)	70% fraction (1 mg)
Number of seeds germinated	10	10	10
% of Control	--	100	100
Radicle length, mm			
Average	77	57	42
Range	64-92	46-77	31-56
Number of seedlings/ number planted	10/10	9/10	10/10
% of Control	--	82	95
Hypocotyl length, mm			
Average	27	15	20
Range	11-39	11-17	16-24
Number of seedlings/ number planted	10/10	5/10	7/10
% of Control	--	56	74
Number of lateral roots			
Average	13	14	12
Range	11-15	13-16	11-14
Number of seedlings/ number planted	9/10	5/10	7/10
% of Control	---	113	100

compounds could possibly be coumarins. Therefore, pure esculin, an available coumarin, was tested for biological activity. Table 10 shows that esculin did inhibit plant growth of C. sativus, but did not affect germination. Degree of inhibition was not proportional to the concentration of esculin used. However, in all cases lateral root growth was the least inhibited and radicle growth was most inhibited. This was the same pattern shown by the 70% MeOH-benzene fraction, however TLC and fluorescence studies did not show a favorable comparison for the two compounds (materials and methods). In general, the rate of germination was stimulated by the presence

Table 9. Growth of *L. sativus* seedlings exposed to acidified 60% MeOH-benzene fraction and 70% MeOH-benzene fraction. Total germination time was 5 days.

Treatment	Control	70% fraction 1 mg	Dissolved portion from 2nd column 60% fraction, 5 mg
Number of seeds germinated	10	1	10
% of Control	--	10	10
Radicle length, mm			
Average	105	60	10
Range	85-130	4 -72	1 -11
Number of seedle/ number plants	8/10	1 /10	6/10
% of Control	--	57	10
Hypocotyl length, mm			
Average	45	41	42
Range	3 -71	37-4	32-6
Number of seedle/ number plants	10/10	6/10	9/10
of Control	--	35	1 5
Number of lateral roots			
Average	10	10	10
ange	1 -12	1 -2	10-
Number of seedle/ number plants	1 /10	10/10	10/10
% of Control	--	100	10

of exogenous although no direct correlation could be made between exogenous concentration and stimulation or germination, i.e.,

As can be seen in Table 11 the aqueous ethanol extract inhibited both germination and growth of *L. sativus* seedlings.

Maximum germination for *L. sativus* controls occurred between 35 and 55 hours of germination. Between 35 hours of age, presence of cytochrom in growth chamber caused inhibition of germination from 35 to 144 hours of age, the number of ungerminated

Table 10. [illegible] of A. [illegible] to [illegible] under [illegible] conditions. Total germination time was 6 days.

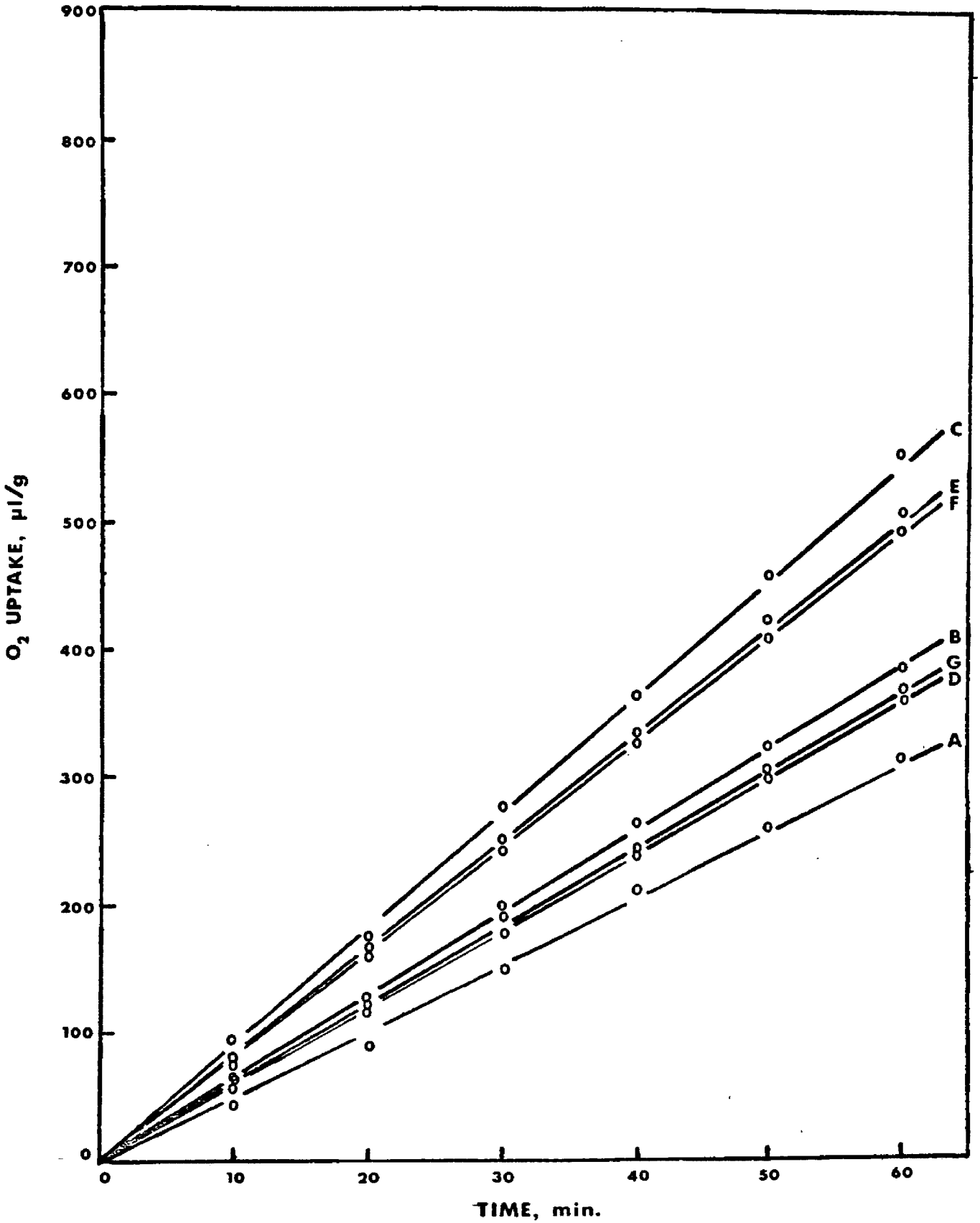
[illegible], %	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Number of seeds germinated	10	10	10	10	10	10	10
% of Control	--	100	100	100	100	100	100
Radicle length, mm							
Average	128	53	61	46	33	23	23
Range	111-146	43-71	37-[illegible]	[illegible]-[illegible]	19-[illegible]	17-[illegible]	16-[illegible]
Number of sample/ number planted	7/10	6/10	5/10	[illegible]/10	10/10	10/10	7/11
% of Control	--	41	48	36	26	18	18
Hypocotyl length, mm							
Average	[illegible]	[illegible]	41	[illegible]	[illegible]	15	[illegible]
Range	55-83	42-56	38-46	19-[illegible]	22-[illegible]	[illegible]-[illegible]	14-31
Number of sample/ number planted	9/10	3/10	6/10	10/10	10/10	10/10	10/11
% of Control	--	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible] of lateral roots							
Average	27	17	32	17	13	11	16
Range	21-31	12-[illegible]	20-[illegible]	15-19	11-[illegible]	7-[illegible]	[illegible]-12
Number of sample/ number planted	[illegible]/10	[illegible]/10	[illegible]/10	[illegible]/10	[illegible]/10	[illegible]/10	7/11
% of Control	--	67	[illegible]	63	[illegible]	[illegible]	[illegible]

Table 11. Growth of A. tridentata ssp. wyomingensis seedlings [illegible] to [illegible] conditions. Total [illegible] time was 6 days.

[illegible]	Roots	[illegible], [illegible]
[illegible] of [illegible]	[illegible]	[illegible]
% of Control	--	[illegible]
Radicle length, mm[a]	[illegible]	[illegible]
% of Control	[illegible]	[illegible]
Hypocotyl length, mm[a]	[illegible]	[illegible]
% of Control	[illegible]	[illegible]

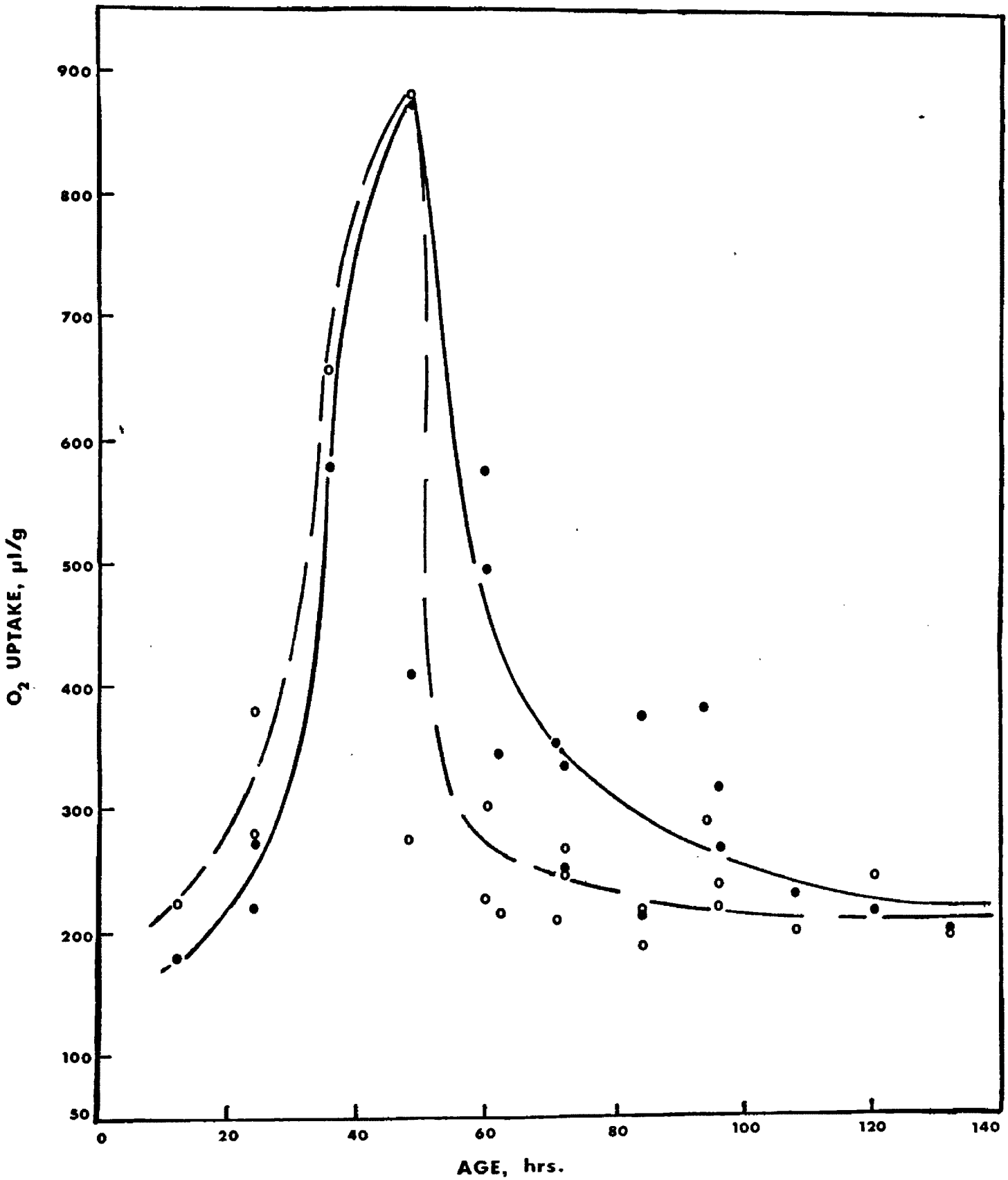
[a] Average length of 10 plants/[illegible].

Figure 4. Rate of oxygen uptake by excised radicles of Cucumis sativus as affected by various amounts of esculin in solution. Radicles were excised from 6-day old seedlings. A - control; B - 1 mg; C - 5 mg; D - 10 mg; E - 15 mg; F - 20 mg; G - 25 mg esculin.



[illegible]

Figure 5. Total oxygen uptake over a thirty hour time period by excised radicles of Cucumis sativus. Radicles were excised from seedlings of various ages. Respiration flasks were paired for each age: one containing no sagebrush, the other containing 0.15 g dried, macerated sagebrush. ● - with sagebrush (solid line); ○ - without sagebrush (dashed line).



CHAPTER IV

SUMMARY

Growth of C. sativum was inhibited by the presence of A. tridentata ssp. vaseyana as shown in Tables 2 through 9. Fresh macerated A. tridentata leaves reduced growth of seedlings to a greater extent than did dried macerate. The known inhibitors camphor and cineole (16) are present in fresh sagebrush and might account for its greater amount of inhibitory activity. However, macerated leaves exhibited inhibitory activity even after drying. This indicates that non-volatile compounds were also active as plant growth inhibitors.

The aqueous methanol extract of sagebrush leaves caused inhibition of growth in proportion to the amount present. This extract was also the one which most significantly reduced germination. Because of this activity, the aq. MeOH extract was chosen for further purification by column chromatography.

Fractions from the column showed both stimulatory and inhibitory biological activity. Fractions which stimulated growth were not studied. The 60% and 70% MeOH-benzene fractions which exhibited inhibitory activity were subjected to further analytical measures. Thin layer chromatography and fluorescence studies suggested that the 60% MeOH-benzene fraction possibly contains a coumarin. Although the 70% MeOH-benzene fraction has a polarity similar to that of the coumarins, it did not show the fluorescence typical of coumarins. Cochromatography with authentic, a known

coumarin found in A. tridentata, showed that these fractions were not esculin. TLC analysis of the 60% MeOH-benzene fraction gave only one broad band and melting points covered broad ranges of temperature. Both tests indicated that further purification is necessary.

It was found that the toxins tested inhibited growth of plant organs differently, especially esculin. With esculin as the growth inhibitor, radicle growth was reduced the least and lateral root growth was inhibited the least for all concentrations of esculin tested.

In all instances of growth reduction, stimulation of respiration was evident. However, it was also found that from 0 to 35 hours of age, C. sativus seedling respiration was inhibited by the presence of sagebrush leaf macerate and that from 35 to 144 hours of age, seedling respiration was stimulated. All instances of growth reduction reported herein were measured from seedlings of 36 to 144 hours of age which showed stimulation of respiration.

Growth inhibition found together with inhibition of respiration indicates that there is interference with the metabolic pathway of the experimental organism. Growth inhibition coupled with stimulation of respiration suggests that an energy uncoupling reaction is reducing the amount of available ATP.

The aqueous methanol extract was shown to inhibit both germination and growth of A. tridentata ssp. wyomingensis. Since A. tridentata ssp. vaseyana and A. tridentata ssp. spiciformis are very closely related, autotoxic effects may be considered a possibility.

Allelopathy is a known phenomenon and, since A. tridentata ssp. vaseyana has been shown to inhibit the growth of C. sativus and, even more severely, the growth of A. tridentata ssp. wyomingensis, it can be suggested that A. tridentata ssp. vaseyana exhibits allelopathic activity in the field. However, the study of allelopathic phenomena must include the consideration of other environmental factors. The plant must be shown to have toxic effects on species native to its habitat. Lab studies and field studies must be correlated with regard to presence, concentration and transportation mechanisms of the allelopathic compound. Other possibilities such as growth limitation by forming end seals, climatic conditions and soil type must also be considered.

In this study, not only volatile compounds, but non-volatile substances were indicated as possible allelopaths. Also, previous studies (1) found inhibition of respiration accompanying inhibition of growth, whereas this study opens the possibility of an uncoupling mechanism, since plants exhibit stimulation of respiration along with inhibition of growth. Tracing inhibitory activity through plants' extracts to find purified compounds which are active is a technique that could be explored further. Indications that biologically active compounds are specific to plant organs provide a lead to determine type and site of inhibition. These advances broaden the scope of investigation of allelopathic activity.

Many questions have been opened for discussion. Fractions which have been found to be active must be purified and iden-

vidual compounds must be identified. Continuation of the research could include several problems. One would be the determination of the type of inhibition, i.e., enzyme inhibition or uncoupling mechanism in respiration, determination of the specific site of inhibition, and elucidation of the form the inhibitor takes within the inhibited plant. Another study, as in the follow-up work Fuller and Fuller conducted, would be to check field conditions as they exist and methods of transport of the compound in the field. A study could be done on the column fractions which appear to stimulate growth. As a practical consideration, studies could be carried out concerning the possibilities of developing "natural" herbicides.

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